NUTRITION OF CANDIDA ALBICANS, CRYPTOCOCCUS [redacted] AND BLASTOMYCES DERMATITIDIS

by

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INTRODUCTION

There is need for additional basic fundamental knowledge of the nutrition and metabolism of pathogenic fungi. A vast amount of knowledge has accumulated on the nutrition, metabolism and enzyme systems of certain bacteria, but little knowledge has accumulated on similar studies of pathogenic fungi.

Variation in morphology and sporulation of fungi, when grown on different media, has been emphasized by Conant (1936). The use of a chemically defined medium would make it possible to study fungi in environments where the number of variables is reduced. The morphological characteristics and physiological activities of a given fungus could then be referable to a standard set of conditions that could be easily duplicated. Results obtained under such conditions would be more reliable and would help to better understand the basic metabolic activities and reasons for morphological changes in fungi.

Chemotherapy and immunization have made little headway in the treatment of diseases due to pathogenic fungi. A better understanding of the physiology and biochemistry of these agents would assist in the testing of fungicides. It would enable the testing of these agents with reference to their metabolic requirements, against suitable enzyme

inactivators.

The purpose of this investigation is to study the nutritional requirements of dimorphic and monomorphic types of pathogenic fungi. The organisms selected for this research were Candida albicans (Monilia albicans), Cryptococcus neoformans (Torula histolytica) and Blastomyces dermatitidis.

REVIEW OF LITERATURE

Early observations in medical mycology were reported chiefly by clinicians. These studies were largely descriptive and morphological. During the past ten years, a few scattered reports have appeared on the physiology and biochemistry of the pathogenic fungi. This is a hopeful indication of increased interest and activity in the field.

Carbon Requirements

Those carbon compounds that can be oxidized with the least expenditure of the energy constitute the food of first choice for fungi. Diversity in ability to use carbohydrates is indicated by numerous reports, but in general the literature shows that glucose is the most readily utilized.

Goddard (1934) showed that glucose, mannose, fructose, maltose, arabinose, and to some extent, sucrose cause increased growth of Tricophyton interdigitale and Microsporon lanosum. Galactose increases the growth of T. interdigitale but not of M. lanosum. Lactose is not used by either species.

Steinberg (1939) lists sucrose, glucose, or fructose as excellent sources of carbon; mannose, maltose, xylose, manitol, and glycerol as fair sources; and galactose and

lactose as generally poor sources of carbon.

Baker and Smith (1942) tested over 40 compounds as a source of carbon for Coccidioides immitis. Sucrose, lactose, cellulose and the sodium salts of formic, butyric, oxalic, tartaric, gluconic and citric acids are not utilized. However, the salts of these acids were not toxic because spores in the inoculum germinated and showed a slight development.

Bacterial metabolism studies show that in the presence of a carbohydrate a decrease in the pH of the medium accompanies the utilization of that carbohydrate, but, according to Goddard (1934) and Stewart and Meyer (1938), this is not true for Trichophyton interdigitale, Coccidioides immitis and Blastomyces dermatitidis. Their experiments showed that even in the presence of quantitative evidence of glucose assimilation the alkalinity of the medium increases. Therefore, the validity of applying bacterial procedures and concepts to the identification of these fungi is questioned.

Nitrogen Requirements

Various workers have shown that great diversity and versatility exists among the fungi in respect to their ability to utilize different nitrogen compounds as their sole source of nitrogen. Robbins (1937) divided fungi into four groups when classified on the basis of their nitrogen requirements. Group one was that group of nitrogen-fixing

organisms that utilize nitrate, ammonium, and organic nitrogen. The second group consisted of those which were incapable of assimilating gaseous nitrogen, but thrived on nitrate-nitrogen, as well as ammonium and organic nitrogen. Those which could grow only with ammonium or organic nitrogen in the medium composed the third group. The fourth group included those fungi which could grow with organic nitrogen alone. The groups were referred to as nitro-fixing nitrate, nitrate, ammonium, or organic nitrogen users.

Peptone, amino acids, amides, ammonium ion and nitrate ion were shown by Baker and Smith (1942) to be suitable as the sole sources of nitrogen for Coccidioides immitis when added to a medium containing salts and glucose. Potassium nitrite did not support the growth of this organism. Stewart and Meyer (1938) obtained good growth of Coccidioides immitis with ammonium salts, acetamide and amino acids, fair growth with urea and poor growth with nitrates as sources of nitrogen. Hirsch and Benson (1927) demonstrated that ammonium lactate served as a source of carbon and nitrogen for this organism. Stewart and Meyer (1939) found that amino acids served as both carbon and nitrogen sources. Urea was a fair source of nitrogen but could not serve as a carbon and nitrogen source at the same time. Roessler, et al. (1946) reported that amino acids, which included all those of known significance in bacterial nutrition, did not improve

the growth of Coccidioides immitis when added singly to a basal medium.

The nitrogen metabolism of Trichophyton mentagrophytes was investigated by Robbins and Ma (1945). This dermatophyte was unable to use ammonium nitrate but could use any one of fourteen amino acids as a source of nitrogen. Combinations were always better for growth due to direct assimilation. The fungus could transform a single amino acid such as asparagine into all the various amino acids necessary for the synthesis of its protein. Goddard (1934) found that T. mentagrophytes could use both nitrates and ammonium salts as sources of nitrogen. While studying the nitrogen and growth factor demands of both the yeast and mycelial phases of Histoplasma capsulatum, Selvin (1949) was able to obtain luxuriant growth of the mycelial phase on a medium with ammonium chloride, ammonium sulfate, or ammonium nitrate as a source of nitrogen. At no time was mycelial development observed on a medium containing nitrite as the sole source of nitrogen. These compounds did not support the development of the yeast-like phase. No single amino acid was necessary for the growth of the mycelial and yeast phase of H. capsulatum.

Vitamin Requirements

Studies on nutritional factors required for growth of yeast have resulted in outstanding contributions to our

knowledge of vitamins. Wilders (1901) showed that an unidentified substance extracted from yeast furnished the necessary stimulus for growth of small inocula of some microorganisms planted in a medium which was in all other respects chemically defined. He called the unknown substance "bios". The possible relationship of vitamins to the growth of yeasts eventually stimulated great interest in vitamins. Copping (1929) showed that yeasts vary considerably in the "bios" requirements. The reviews of growth promoting requirements by Williams (1941), Snell (1946) and Tatum (1944) present a clear picture of recent developments in this field. It is now recognized that "bios" is actually a mixture of substances required by different strains of yeasts.

Williams and Rohrman (1936) maintained that minimum complement of growth accessory factors required by yeast includes aspartic acid, pantothenic acid, inositol, and thiamine. Burkholder, et al. (1944) made an extensive study of some of the growth factors of yeast. Among the 86 named kinds of yeast found not to be heterotrophic, one or more vitamin deficiencies occurred as follows: biotin 78, thiamine 33, pantothenic acid 30, inositol 15, nicotinic acid 13, and pyridoxine 13. No deficiency for riboflavin was observed. Richards (1936) showed that small amounts of pantothenic acid concentrate produce a marked increase

in the growth of a pure strain of Saccharomyces cerevisiae. The presence of the pantothenic acid increased the yeast growth by lessening the generation time. The increase was more pronounced when the seed came from old cultures.

Rogosa (1943) working with several different yeasts found that those that fermented lactose needed nicotinic acid to satisfy normal growth while those yeasts that do not ferment lactose readily synthesize sufficient nicotinic acid for optimal growth. Van Lanen (1947) found that niacin is taken up from the medium by both growing and fermenting yeast with less avidity than is thiamine.

Burkholder (1943) found that Candida albicans appeared to need biotin for growth. All of the strains of Trichophyton mentagrophytes and Triphophyton rubrum studied grew well without the addition of any vitamin. Trichophyton acuminatum, Trichophyton sulfureum, Trichophyton violaceum, Hoemodendron Pedrosoi, Phialophora verrucosa, Sporotrichun Schenckii were all reported to be deficient for thiamine. Trichophyton faviforme was reported as being deficient for thiamine and inositol.

Selvin (1949) reported that biotin was the only growth factor necessary for the development of the yeast-like phase of Histoplasma capsulatum but this vitamin had no effect on the mycelial growth of the fungus.

Robbins and Kavanagh (1943) have summarized the literature

up to 1942 on the relation of vitamins to the growth of specific fungi. They indicated that although few of the pathogenic fungi had been studied, growth of Microsporum fulvum, Trichophyton crateriforme, Trichophyton rosaceum, Cryptococcus (Busse-Buschke) and Sporotrichum Schenckii in a synthetic medium was stimulated by the addition of either yeast extract, rice polishings, or thiamine. Mager and Aschner (1946) showed that vitamins other than thiamine were not essential for cultivation for starch producing yeast in synthetic media. Reid (1949) studied the influence of the vitamin B complex on the growth of Cryptococcus neoformans on a solid synthetic medium. He found that thiamine was the only member of the vitamin B group which was stimulatory to the growth of this fungus. A strain of Trichophyton equinum was found by Georg (1949) to require nicotinic acid. Comparatively large amounts of l-tryptophane could be substituted for the vitamin.

Trace Element Requirements

The essential role heavy metals plays in nutrition of molds has been recognized since the earliest investigations dealing with the cultivation of fungi in synthetic media. The amounts of heavy metal ions which are effective in obtaining maximum growths of fungi are so minute as compared with the more familiar mineral constituents of

artificial media that the term "trace elements" is often applied to them. In the cultivation of fungi for routine isolation, the requirements for trace elements are ignored but from a physiological and biochemical viewpoint, further knowledge of trace element nutrition is needed.

Organisms with simple growth requirements present difficulties perhaps not fully appreciated by workers whose experience has been with forms having complicated organic requirements. Chief among the obstacles in this field is the lack of a medium sufficiently free from a trace element to reveal marked deficiencies. The few methods available utilize the principles of recrystalization, precipitation, or adsorption. Precipitation methods, according to Waring and Werkman (1943), are not successful because the precipitations are not complete in a biological sense. A medium that is "chemically free" from iron may still contain the metal in concentrations two or three orders of magnitude greater than that "biologically free" from the element.

Many of the trace elements which are essential in extremely small amounts are present as contaminants in chemical constituents of media. Hutner (1946) worked on the growth essentials of the photosynthetic bacteria. He found that his lactate was contaminated with growth stimulating elements. Many other workers have concurred with

Hutner on the difficulties in the investigation of mineral requirements.

Steinberg (1939) showed that iron, zinc, copper, manganese, molybdenum and gallium are needed to support the growth of Aspergillus niger. Roessler, et al. (1946) investigated the nutrition of Coccidioides immitis in a chemically defined medium and found that magnesium was needed for the growth of this organism. In cultures containing zinc sulfate, the growth was initiated sooner, grew more rapidly, and produced slightly more growth than did a control culture. Trichophyton mentagrophytes (interdigitale) was shown to require calcium in its nutrition by Mosher, et al. (1936). Iron zinc, copper and manganese were found also to be beneficial for the growth of this organism.

Nickerson and Chadwick (1946) published a report on the effect of the inorganic salts of zinc, mercury, and cadmium upon the respiration of three dermatophytes. Salts of mercury, silver, and zinc depressed respiration while cadmium compounds had little effect.

Hydrogen Ion Concentration

Pathogenic fungi are not very exacting in their hydrogen ion requirements. The pH limits of growth have been determined for only a few of these organisms. Peck and Rosenfeld (1938) showed that Trichophyton gypseum, Epidermophyton

inguinale and Candida albicans grew between pH 4.0 to 9.0. Biltres (1929) has shown that in peptone solutions Trichophyton gypseum grew within the limits from pH 3.7 to pH 11. When the initial pH was below 8 the medium became alkaline with the growth of the fungus. Leise and James (1945) have made use of the ability of dermatophytes to grow well under alkaline conditions in devising a medium buffered to pH 10.5 for the isolation of dermatophytes. On a series of buffered corn meal agar media inoculated with Trichophyton rubrum, Jillson and Nickerson (1948) obtained good growth within the pH range of 4.5 to 8.0. Karnaky (1945, 1946) found that a pathogenic strain of Candida albicans grew profusely in Sabouraud's medium from pH 3.9 to 10.8. Buffers retarded the growth slightly, but this may have been due to the action of preventing the organism from influencing the pH.

The effect of the hydrogen-ion concentration on the mycelial phase of Histoplasma capsulatum has been studied and reported by Howell (1941). He found that the hydrogen-ion concentration affects growth and sporulation. The optimum hydrogen-ion concentration may vary with the medium used. Selvin (1947) reported that the yeast-like phase of Histoplasma capsulatum in a fluid medium grew best at hydrogen-ion concentrations between 6.3 and 8.1. Cross (1943) showed that the optimal initial hydrogen-ion

concentration for the growth of the yeast-like phase of Histoplasma capsulatum was between pH 7.2 and 7.6.

Levine and Ordal (1946) have shown that pH is unimportant in the conversion of Blastomyces dermatitidis from one phase to the other.

MATERIALS AND METHODS

Pyrex glassware was used exclusively in this study. Tubes and glass equipment were soaked in dichromate-sulfuric acid solution. They were then washed with tap water and rinsed five times with distilled water. The last rinsing was with triple distilled water.

In the preparation of media, the best grade of chemicals available was used. Solutions and media were prepared with triple distilled water.

Organisms

Blastomyces dermatitidis is the causitive agent of North American blastomycosis, or Gilchrist's disease. It exists in man and in experimentally infected animals in single celled, budding yeast-like states. In culture the organism may be maintained in the yeast form if incubated at 37° C., or in a filamentous, mycelial form if incubated at room temperature. The strains investigated, were Blastomyces dermatitidis No. 9533, from the American Type Culture Collection, Tulane* Strain No. 380, and Tulane* Strain No. 410.

Candida albicans is best known as the causitive agent of thrush, a disease of the throat and mouth of children.

*The Tulane strains of Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis were obtained from Dr. M. L. Littman, Department of Tropical Medicine and Public Health, Tulane University School of Medicine, New Orleans, Louisiana.

Other infections caused by this organism are cutaneous moniliasis and bronchopulmonary moniliasis. It exists in the body as small, oval, budding thin-walled yeast cells. On cultural media the growth is usually yeast-like during the first stages of multiplication, but may, under some conditions, later develop pseudomycelial and mycelial elements which may terminate with large chlamydospores. The strains used in this investigation were Candida albicans from the stock collection of the University of Utah, Salt Lake City, Utah, Candida albicans Tulane Strain No. 520, and a strain isolated from a patient with a pulmonary infection, from the Veterans' Hospital, Salt Lake City, Utah. This strain proved to be pathogenic for mice and rabbits.

Cryptococcus neoformans, the causitive agent of pulmonary cryptococcosis and a fatal type of meningitis, was included in this study. This organism grows in the yeast-like phase in the body and in cultural media. The strains investigated were, a stock culture maintained by the Bacteriology Department, University of Utah, Tulane Strain No. 823, and Tulane Strain No. 835.

Media

Stock cultures of the organisms were maintained on Sabouraud's maltose agar. The yeast-like phase of Blastomyces dermatitidis was maintained on blood agar

Hydrogen ion concentration. The pH of the media was determined before autoclaving with a Beckman glass electrode potentiometer. Except where it was being used as a variable, the pH was adjusted to 5.6 to 5.8.

Trace element solution. A stock solution of the trace elements used in Wickerham's media (1946) was prepared 100 times the concentration desired in the medium. This stock solution was stored in a glass stoppered bottle.

Vitamins. Solutions of vitamins were made up in quantities of 100 times the final concentration in triple distilled water. The solution of thiamine hydrochloride was sterilized by filtration through a Seitz filter. The remaining vitamin solutions were sterilized in the autoclave at 120° C. for 15 minutes. The solutions were stored in screw cap bottles in the refrigerator for use as needed. Freshly made vitamin solutions were used in the experiment on vitamin requirements.

Inoculum and Incubation

The inoculum of Candida albicans and Cryptococcus neoformans was grown for 24 hours at room temperature in a synthetic medium. The organisms were then washed with sterile distilled water three times and suspended in sterile distilled water. The yeast-like phase of a four day old culture of Blastomyces dermatitidis was used in

the preparation of the inoculum.

The suspensions of the inoculum were standardized to a density reading of 50 on the Klett-Summerson photoelectric colorimeter using a number 42 filter. One drop of the suspension delivered from a one ml pipette was used to inoculate each tube of medium. The tubes were shaken after inoculation to suspend the cells uniformly.

Incubation. The incubation of Candida albicans and Cryptococcus neoformans was carried out at room temperature (18° to 28° C.). Two temperatures were used for the incubation of Blastomyces dermatitidis, 37° C. for the observation of the yeast-like phase and room temperature for growing the organism in the mycelial phase.

Measurement of Growth

The extent of growth in media inoculated with Candida albicans and Cryptococcus neoformans was measured with a Klett-Summerson photoelectric colorimeter using a No. 42 filter. These turbidity readings are reported as the average of triplicate tubes of a single experiment.

The growth of Blastomyces dermatitidis in the various media was visually compared with control tubes.

EXPERIMENTAL RESULTS

Carbon Assimilation

The ability of Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis to utilize different carbon compounds was investigated by the method of Wickerham and Burden (1948). The medium consisted of trace mineral salts, salts, ammonium sulfate, 8 vitamins, and glucose. The concentrations of the constituents of the medium are given in Table 1. Various carbon compounds were substituted for glucose. The final concentration of the lactic, malic, fumaric, pyruvic, succinic and citric acids was 0.25 per cent. The rest of the carbon sources were used in a concentration of 0.5 per cent.

The media which contained lactic and pyruvic acids as a carbon source were sterilized by passage through a Seitz filter. The other media were sterilized in an autoclave at 120° C. at 15 lbs. pressure for 15 minutes. Ethyl alcohol was added to the medium after sterilization.

The inoculum for this experiment was grown on Sabouraud's agar slants. A transfer of the culture was made into the Wickerham medium containing as carbohydrate 0.1 per cent glucose. This culture was diluted with basal medium containing no carbohydrate. The incubation time of media inoculated with Candida albicans, Cryptococcus neoformans was 14 days. Blastomyces dermatitidis cultures were incubated for 21 days.

Table 1

Composition of Wickerham Medium

Compound	Concentration / liter
Trace Elements	
Boron as H_3BO_4	0.01 ppm
Copper as $CuSO_4$	0.01 ppm
Iodine as KI	0.10 ppm
Iron as $FeCl \cdot 7H_2O$	0.05 ppm
Zinc as $ZnSO_4 \cdot 7H_2O$	0.07 ppm
Manganese as $MnSO_4 \cdot 4H_2O$	0.10 ppm
Molybdenum as $Na_2MoO_4 \cdot 2H_2O$	0.01 ppm
Vitamins	
Biotin	2 ug
Ca. Pantothenate	200 ug
Inositol	2,000 ug
Niacin	200 ug
Para-aminobenzoic acid	200 ug
Pyridoxine hydrochloride	200 ug
Thiamine hydrochloride	200 ug
Riboflavin	200 ug
Salts	
Ammonium sulfate	1.00 gram
Potassium acid phosphate	1.00 gram
Magnesium sulfate	0.50 gram
Sodium chloride	0.10 gram
$CaCl_2 \cdot 2H_2O$	0.10 gram
Glucose	10.00 grams

The results of this investigation are shown in Table 2.

Glucose, maltose, sucrose, galactose, levulose, d-xylose, trehalose, d-manitol, succinic acid, dl-lactic acid, and pyruvic acid were assimilated giving heavy growth of Candida albicans. Ten carbon compounds, inulin, soluble starch, adonitol, sorbitol, malic acid, fumaric acid, citric acid, acetic acid, ethyl alcohol, and ethyl acetate, gave fair growth of the organism. Slight growth was obtained with raffinose, arabinose and glycerine. Five of the compounds, lactose, ribose, rhamnose, cellobiose and dulcitol, failed to support the growth of Candida albicans.

Cryptococcus neoformans utilizes maltose, sucrose, levulose, inulin, manitol, and sorbitol giving a density reading comparable to that of glucose. Galactose, raffinose, xylose, arabinose, rhamnose, trehalose, soluble starch, malic and citric acids, were intermediate in their ability to support the growth of this organism. Slight growth was observed in the media with ribose, cellobiose, adonitol or lactic acid as the carbon source. No growth appeared when lactose, dulcitol, fumaric acid, acetic acid, glycerine, or ethyl acetate were the carbon sources.

Little difference existed in the ability of the yeast-like and mycelial phases of Blastomyces dermatitidis to utilize different carbon compounds. Good sources of carbon for this organism are glucose, maltose, sucrose, raffinose, fumaric acid, succinic acid and glycerine. Fair growth was

Table 2.

Utilization of Carbon Compounds by Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis

Carbon Compound	Candida albicans*	Cryptococcus neoformans*	Blastomyces dermatitidis** (room temp.)	Blastomyces dermatitidis** (37° C.)
Basal	3	7	0	0
Glucose	189	118	++++	++++
Maltose	278	230	++++	++++
Lactose	0	3	0	0
Sucrose	172	120	+++	+++
Galactose	181	70	+	+
Levulose	155	105	0	0
Raffinose	20	74	+++	+++
D-Xylose	142	75	0	0
L-Arabinose	23	54	++	++
D-Ribose	3	36	0	0
L-Rhamnose	2	68	0	0
Cellobiose	8	24	++	++
Trehalose	174	48	++	++
Inulin	65	155	+	+
Soluble Starch	78	73	++	++
Adonitol	65	38	0	0
Dulcitol	1	2	+	+
D-Mannitol	224	129	+	+
D-Sorbitol	104	125	++	++
Malic Acid	70	67	++	+
Fumaric Acid	96	9	+++	+++
Succinic Acid	142	42	+++	++
DL-Lactic Acid	194	18	++	++
Pyruvic Acid	177	14	++	++
Citric Acid	106	71	+	+
Acetic Acid	73	5	+	+
Glycerine	23	10	+++	+++
Ethyl Alcohol	119	30	+	+
Ethyl Acetate	52	6	0	0

* Density of growth measured with a Klett-Summerson photoelectric colorimeter.

** Growth in various mediums is compared with control tubes containing basal medium but no carbon source. ++++ = heavy growth, +++ = good growth, ++ = fair growth, + = slight growth, 0 = no growth.

obtained in the media with arabinose, cellobiose, trehalose, soluble starch, malic acid, lactic acid, sorbitol, or pyruvic acid as the carbon source. Slight growth was obtained in those media which contain galactose, inulin, dulcitol, manitol, citric acid, acetic acid, or ethyl alcohol as their source of carbon. Blastomyces dermatitidis is unable to utilize lactose, levulose, xylose, ribose, rhamnose, adonitol, and ethyl acetate as a source of carbon.

Nitrogen Assimilation

The ability of Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis to assimilate various nitrogen containing compounds was tested by the method of Wickerham (1948). The medium used in this experiment was the same as that used in the carbon assimilation experiment and is given in Table 1. Different nitrogen compounds were substituted for ammonium sulfate in the concentrations listed in Table 3.

Table 3.

Concentration of Nitrogen Compounds

Compound	Grams / l
Ammonium chloride	1.00
Potassium nitrate	0.78
Urea	0.46
Asparagine	1.00
Peptone	1.32

The urea medium was sterilized by passage through a Seitz filter. It was then dispensed aseptically in 5 ml amounts into sterile culture tubes. The other media, after tubing, were sterilized in the autoclave at 15 lbs. pressure for 15 minutes. The previously described procedure of inoculation and incubation was followed out. The media inoculated with Candida albicans and Cryptococcus neoformans was incubated for 14 days. Blastomyces dermatitidis cultures were left for 21 days. The measurement of growth is recorded in Table 4.

Potassium nitrate was the only nitrogen compound tested which did not support the growth of these fungi. There was a lower density of growth of Candida albicans in the urea medium, but the concentration of the nitrogen was much lower. Growth of Cryptococcus neoformans was not as heavy as that of Candida albicans. The urea medium supported the growth of this organism comparable to the other media.

Both the yeast-like and mycelial phase of Blastomyces dermatitidis assimilated ammonium sulfate, ammonium chloride, peptone, asparagine and urea, but not potassium nitrate.

Amino Acid Assimilation. A series of media containing a combination of different amino acids were prepared. Ten mg of each of the following crystalline amino acids was included in the medium as the nitrogen source: glycine, dl-alanine, dl-valine, dl-leucine, dl-isoleucine, dl-serine, dl-threonine, dl-phenylalanine, dl-tryptophane, l-cystine,

Table 4.

Assimilation of Nitrogen Sources by Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis.

Medium	Candida albicans*	Cryptococcus neoformans*	Blastomyces dermatitidis** (room temp.)	Blastomyces dermatitidis** (37° C.)
Basal	12	6	0	0
Ammonium Sulfate	204	94	ƒƒƒ	ƒƒƒ
Ammonium Chloride	190	85	ƒƒƒ	ƒƒƒ
Potassium Nitrate	9	3	0	0
Peptone	280	104	ƒƒƒƒ	ƒƒƒƒ
Asparagine	264	108	ƒƒƒ	ƒƒƒ
Urea	130	111	ƒƒƒ	ƒƒƒ

* Density of growth measured with a Klett-Summerson photoelectric colorimeter.

** Growth in various mediums is compared with control tubes containing basal medium but no nitrogen source. ƒƒƒƒ = heavy growth, ƒƒƒ = good growth, ƒƒ = fair growth, ƒ = slight growth, 0 = no growth.

dl-methionine, dl-aspartic acid, l-glutamic acid, l-lysine, l-arginine, l-histidine, and l-proline. Single omissions of each of the different amino acids were made from the media.

Heavy growth developed in all the media inoculated with the fungi. There were very little differences existing between the tubes. No single amino acid is essential for the growth of these organisms.

Tubes of media were prepared in each of which 1.8 grams per liter of a different single amino acid were included. The same procedure of inoculation and incubation as previously described were followed. The results are shown in Table 5.

All the amino acids tested supported the growth of Candida albicans. Best growth was attained with isoleucine, and the poorest growth was in the medium that contained threonine as the nitrogen source. Cryptococcus neoformans showed poor growth in the media containing threonine and methionine. Slight growth was found in the media with leucine, isoleucine and lysine as nitrogen sources. Valine, serine, tryptophane and cystine gave fair growth. The most prolific growth was in the media that contained proline, aspartic acid, glutamic acid and alanine. Table 5 shows that in the tubes inoculated with the yeast-like phase of Blastomyces dermatitidis, glycine, alanine, serine, aspartic acid, glutamic acid and proline gave the best growth. This was true for both the mycelial and yeast-like phases of this organism. The media that contained leucine, threonine, cystine, methionine, lycine, and histidine didn't support

Table 5.

Growth of Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis on Synthetic Media Containing a Single Amino Acid as Nitrogen Source

Amino Acid	Candida albicans*	Cryptococcus neoformans*	Blastomyces dermatitidis** (room temp.)	Blastomyces dermatitidis** (37° C.)
Basal	7	4	0	0
Glycine	207	105	++++	++++
dl-Alanine	210	142	++++	++++
dl-Valine	223	63	++	++
dl-Leucine	187	46	+	0
dl-Isoleucine	239	51	++	+
dl-Serine	228	79	++++	++++
dl-Threonine	135	20	+	0
dl-Phenylalanine	197	102	+	0
dl-Tryptophane	195	63	++	++
l-Cystine	156	69	+	0
dl-Methionine	168	16	+	0
dl-Aspartic Acid	178	169	++++	++++
l(+)Glutamic Acid	225	142	++++	++++
l(+)Lysine	175	46	+	0
l(+)Arginine	229	110	+	+
l-Histidine	165	96	+	0
l-Proline	190	177	++++	++++

* Density of growth measured with a Klett-Summerson photoelectric colorimeter.

** Growth in various mediums is compared with control tubes containing basal medium but no amino acid source. ++++ = heavy growth, +++ = good growth, ++ = fair growth, + = slight growth, 0 = no growth.

the growth of the yeast-like phase. When growth was present in the tubes incubated at 37° C. it was in the yeast-like phase.

Vitamin Requirements

In order to investigate the vitamin requirements of Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis, a series of different media were prepared. The basal media contained glucose, salts and trace elements. Vitamin supplements were added singly or in combination. The concentrations of the various constituents are given in Table 1.

The kinds of media employed for the testing of the vitamin requirements of each organism is as follows: No addition of vitamins to the basal media, eight vitamins plus the basal medium, single omission of each of the eight vitamins, single vitamins added to the basal medium, and eight vitamins plus 0.1 per cent yeast extract added to the basal medium. One medium contained the eight vitamins and basal medium except the nitrogen source was 0.25 per cent ammonium sulfate and 0.25 per cent l-asparagine.

With the exception of thiamine, sterilization was done in an autoclave at 15 lbs. pressure for 15 minutes. The thiamine solution was prepared 100 times the concentration

and was sterilized by passage through Seitz filter. It was added aseptically to the sterilized media. The different media were pipetted aseptically in 5 ml amounts into sterile culture tubes. The growth densities were measured after 3, 7, and 14 days. Blastomyces dermatitidis cultures were allowed to grow for 21 days.

Table 6 shows the growth obtained with the three fungi at the end of the incubation period.

The basal medium alone allowed Candida albicans a bare minimal growth. The addition of all the 8 vitamins investigated gave a luxuriant growth. Only when biotin was a part of the vitamin supplement did heavy growth appear. When biotin was omitted from the media, the growth fell to that of the medium without any of the vitamins. The growth of Candida albicans in a medium containing biotin was comparable to the growth in the medium which contained yeast extract and 8 vitamins. Table 6 shows the results obtained on three of the media to which the vitamins were added singly. Only the medium containing biotin showed growth stimulation with single additions of the vitamins.

Table 6 shows that the growth of Cryptococcus neoformans is stimulated by thiamine. All media containing thiamine gave growth comparable to the medium with all 8 vitamins. If thiamine was omitted from the medium the growth fell to

Table 6.

Growth of Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis with single omissions and single additions of vitamins

Medium	Candida albicans*	Cryptococcus neoformans*	Blastomyces dermatitidis** (room temp.)	Blastomyces dermatitidis** (37° C.)
Basal Medium	7	1	++++	++++
All Vitamins	335	330	++++	++++
All Vitamins -Thiamine	315	17	++++	++++
-Pantothenic	310	305	++++	++++
-Niacin	325	335	++++	++++
-Biotin	3	335	++++	++++
-Inositol	325	320	++++	++++
-Riboflavin	310	315	++++	++++
-Pyridoxine	340	315	++++	++++
+Yeast Extract	345	375	++++	++++
+Asparagine	350	335	++++	++++
Basal +Biotin	347	4	++++	++++
+Thiamine	2	348	++++	++++
+Pyridoxine	5	2	++++	++++

* Density of growth measured with a Klett-Summerson photoelectric colorimeter.

** Growth in various mediums is compared control tubes containing basal medium. ++++ = heavy growth.

a bare minimum. When thiamine is the only vitamin added to the medium, heavy growth is obtained.

The results of the investigation of the yeast-like and mycelial phase of Blastomyces dermatitidis showed that none of the vitamins tested have any stimulatory effect on the growth of this dimorphic fungi.

Hydrogen Ion Concentration

The purpose of this experiment was to study the effect of the initial hydrogen ion concentration upon the growth of Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis. The composition of the medium was that described in Table 1, except casein hydrolysate in 0.1 per cent concentration was substituted for the ammonium sulfate. Portions of each of these media were adjusted to hydrogen ion concentrations of pH 3.6, 4.2, 4.5, 5.2, 5.7, 6.3, 6.7, 7.3, 7.7, 8.1, 8.5 and 9.0. The pH values were measured with a Beckman glass electrode potentiometer after sterilization in the autoclave at 120° for 15 minutes.

Five ml of each of medium were pipetted aseptically into sterile culture tubes, inoculated and incubated as previously described. After seven days, the growth in the tubes was measured. The results are given in Table 7.

Candida albicans grew in all of the hydrogen ion concentrations tested. Best growth appeared, however, in media

Table 7.

The Effect of Initial Hydrogen Ion Concentration on the Growth of Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis

pH	Candida albicans*	Cryptococcus neoformans*	Blastomyces dermatitidis** (room temp.)	Blastomyces dermatitidis** (37° C.)
3.6	78	98	++	+
4.2	137	135	++	+
4.5	130	128	+++	+
5.2	140	135	++++	+++
5.7	130	142	++++	++++
6.3	120	130	++++	++++
6.7	120	106	++++	++++
7.3	115	112	++++	++++
7.7	105	105	+++	+++
8.1	102	94	++	++
8.5	95	90	++	+
9.0	56	80	+	+

* Density of growth measured with a Klett-Summerson photoelectric colorimeter.

** Comparative growth in the various mediums. ++++ = heavy growth, +++ = good growth, ++ = fair growth, + = slight growth, 0 = no growth.

between pH 4.2 and 6.7 with the optimum pH of 5.2. The optimum hydrogen ion concentration for growth of Cryptococcus neoformans appeared near 5.7, but there was growth in the entire pH range tested. There was a decline of growth at the extremes of the pH range of the tubes inoculated with Blastomyces dermatitidis with the best growth in the range from 5.2 to 7.7.

Synthetic Medium

The synthetic medium given in Table 8 was found to support the growth of the pathogenic fungi under study. Repeated transfers of the organisms were made in this medium.

Table 8.

Composition of Synthetic Medium

Compound	Concentration
Glucose	10.0 grams
Ammonium sulfate	1.00 gram
Magnesium sulfate	0.50 gram
Monopotassium phosphate	1.00 gram
Biotin	2 ug
Thiamine	200 ug
Distilled water	1,000 ml

Biotin could be excluded from the medium when inoculated

with Cryptococcus neoformans and thiamine could be excluded in Candida albicans media.

Neither of the two vitamins were needed for the growth of Blastomyces dermatitidis.

The growth on this media was never as heavy as when the media contained peptone or casein hydrolysate, indicating a stimulation from amino acids.

DISCUSSION

This investigation of the various factors affecting the growth of Candida albicans, Cryptococcus neoformans, and Blastomyces dermatitidis has shown that these organisms are not fastidious in their nutritional requirements. A simple synthetic medium consisting of glucose, ammonium sulfate, phosphate buffer and magnesium sulfate will support the growth of Blastomyces dermatitidis. For the growth of Candida albicans, biotin must be added, and the growth of Cryptococcus neoformans is stimulated with thiamine.

Many different carbon compounds can be substituted for glucose in this medium and still support the growth of these organisms. Various amino acids and nitrogen compounds can be utilized as nitrogen sources.

For many years the assimilation of ammonium sulfate, urea, asparagine, and peptone has been used as an aid in classification of yeast. Wickerham (1946) claimed that such tests were invalid because of the wide number of yeast that could utilize these compounds as nitrogen sources. All the yeasts tested by Wickerham that were previously designated as incapable of assimilating these compounds, utilized the nitrogen compounds when an adequate supply of pure vitamins was added. The results of the present investigation concur with those of Wickerham. Candida albicans, Cryptococcus

neoformans and Blastomyces dermatitidis were not studied by Wickerham. These pathogenic fungi utilized ammonium sulfate, as well as urea, asparagine and peptone as a nitrogen source. However, potassium nitrate was not assimilated.

During the progress of the present investigation, Reid (1949) reported that thiamine was necessary for the growth of Cryptococcus neoformans on a solid synthetic media. The growth of the organism was washed from agar slants prepared from his synthetic medium. Density determinations were made from these suspensions. The present work confirms Reid's observation on a liquid synthetic medium.

The nutritional requirements of Blastomyces dermatitidis in both the yeast-like and mycelial phases are similar. When the yeast-like phase was used as the inoculum, the temperature of incubation determined the phase of growth. If the cultures were left at room temperature the mycelial growth developed. When cultures were incubated at 37° C. the yeast-like phase persisted.

Obviously some phases of the nutritional requirements of these pathogenic organisms have not been studied. Investigations of trace mineral requirements are needed. The organisms will multiply without the addition of trace mineral salts, but these elements are very likely present as contaminents in the reagents used in the preparation of

the medium. Another phase of study that would be desirable is that of the gaseous requirements. When cultures of the pathogenic fungi used in the present study were placed on a shaking machine, oscillating horizontally, visable growth appeared more rapidly than in control flasks that were not shaken. The total cell volume was slightly greater in the shaken flasks than in the control flasks.

SUMMARY

A wide variety of carbon compounds can serve as carbon sources for Candida albicans, Cryptococcus neoformans and Blastomyces dermatitidis. These carbon sources include many carbohydrates, alcohols, and organic acids.

The organisms can assimilate, as nitrogen sources, ammonium sulfate, ammonium chloride, urea, asparagine, or peptone. Potassium nitrate is not utilized by any of these organisms.

Various amino acids can serve as a nitrogen source in a synthetic medium.

Biotin is the only vitamin which must be added to a synthetic medium for the growth of Candida albicans. Cryptococcus neoformans requires thiamine for growth. Blastomyces dermatitidis does not require preformed vitamins added to synthetic medium.

The initial hydrogen ion concentration is relatively unimportant in the ability of these organisms to multiply. All of the fungi under investigation grew in media adjusted over a pH range of 3.6 to 9.0.

A synthetic medium which will support the growth of the organisms under study consists of the following: Glucose 10.0 grams; ammonium sulfate 1.00 gram; magnesium sulfate 0.5 gram; monopotassium phosphate 1.00 gram;

biotin 2 ug; thiamine 200 ug and triple distilled water 1000 ml. Biotin can be excluded from the medium for the growth of Cryptococcus neoformans, and thiamine can be excluded for growth of Candida albicans. Neither biotin nor thiamine are required in the medium for the growth of Blastomyces dermatitidis.

REFERENCES

Baker, E.E. and Smith, C.E. 1942. Utilization of carbon and nitrogen compounds by Coccicioides immitis. J. Infect. Dis. 70: 51.

Biltris, R. 1929. Sur la variabilite des caracteres de l'espece chez les dermatophytes. Ann. Inst. Pasteur 43: 281.

Burkholder, P.R. 1943. Vitamin deficiencies of fifty yeasts and molds. Bul. of the Torrey Botanical Club, 70: 372.

Burkholder, P.R., Moyer, D. and McVeigh, I. 1944. Studies on some growth factors of yeast. J. Bact. 48: 385.

Conant, N.F. 1939. Studies in the genus Microsporum. Arch. of Demat. and Syphilal. 33: 665.

Copping, A.M. 1929. The effect of bios on the growth and metabolism of certain yeasts. Biochem. J. 23: 1050.

Cross, F.W. 1948. The effect of hydrogen-ion concentration on the yeast-like phase of Histoplasma capsulatum (Darling). Public Health Reports 63: 739.

Foster, J.W. 1939. The heavy metal nutrition of fungi. Botanical Review 5: 207.

Georg, L.K. 1949. Conversion of tryptophane to nicotinic acid by Trichophyton equinum. Proc. Soc. Exp. Biol. & Med. 72: 653.

Goddard, D.R. 1934. Phases of the metabolism of Trichophyton interdigitale. J. Infect. Dis. 54: 149.

Hirsch, E.F., and Benson, H. 1927. Specific skin and testes reactions with cultures filtrate of Coccidioides immitis. J. Infect. Dis. 40: 629.

Howell, Arden Jr. 1941 Studies on Histoplasma capsulatum and similar form species. III Effect of hydrogen-ion concentration. Mycologia 33: 103.

Hutner, S.H. 1946. Organic growth requirements of photosynthetic bacteria. J. Bact. 52: 213.

Jellson, O.F. and Nickerson, Walter J. 1948. Mutual antagonism between pathogenic fungi. Inhibition of dimorphism in Candida albicans. Mycologia 40: 369.

Karnaky, K.J. 1945. At what pH does pathogenic Monilia albicans grow best. West. J. Surg., 53: 390.

Karnaky, K.J. 1946. Hydrogen-ion concentration (pH) of Monilia albicans infection and treatment. South Med. J. 39: 731.

Leise, J.J. and James, L.H. 1945. An alkaline medium and procedure for the selection of dematophytes in the presence of saprophytic fungi. J. Lab. Clin. Med., 30: 119.

Levine, S. and Ordal, Z.J. 1946. Factors influencing the morphology of Blastomyces dermatitidis. J. Bact., 52: 687.

Mager, J. and Aschner, M. 1946. Starch reaction as aid in identification of causative agent of "European Blastomycosis". Proc. Soc. Expt. Biol. Med., 62: 71.

Moore, M. and MaPother, P. 1940 Chromomycosis of the face. Arch. Demat. & Syphilol., 41: 42.

Mosher, W.A., Saunders, D.H., Kingery, L.K. and Williams, R.J. 1936. Nutritional requirements of the pathogenic mold, Trichophyton interdigitale. Plant Physiol., 11: 795.

Nickerson, Walter J. and Chadwick, J.B. 1946. On the respiration of dermatophytes. Arch. Biochem., 10: 81.

Peck, S.M. and Rosenfeld, H. 1938. The effect of hydrogen-ion concentration, fatty acids, and vitamin "C" on the growth of fungi. J. Invest. Dermatology, 1: 237.

Reid, J.D. 1949. The influence of the vitamin B complex on the growth of Torulopsis (Cryptococcus) neoformans on a synthetic medium. J. Bact. 58: 777.

Richards, Oscar W. 1936. The stimulation of yeast proliferation by pantothenic acid. J. Biochem., 113: 531.

Robbins, W.J. 1937. The assimilation by plants of various forms of nitrogen. Amer. J. Bot., 24: 243.

Robbins, W.J. and Kavanagh, V. 1942. Vitamin deficiencies of the filamentous fungi. Bot. Rev., 8: 411.

Robbins, W.J. and Ma, R. 1945. Growth factors for Trichophyton mentagrophytes. Am. J. Bot., 32: 509.

Roessler, W.G., Herbst, E.J., McCoullough, W.G., Mills, R.C. and Brewer, C.R. 1946. The nutrition of Coccidioides immitis in submerged culture. J. Infect. Dis., 79: 12.

Rogosa, M. 1943. Nicotinic acid requirements of certain yeast. J. Bact., 46: 435.

Selvin, S.B. 1947. Cultural studies on the yeast-like phase of Histoplasma capsulatum (Darling). J. Bact., 54: 655.

Selvin, S.B. 1949. Cysteine and related compounds in the growth of the yeast-like phase of Histoplasma capsulatum. J. Infect. Dis., 84: 275.

Snell, E.E. 1946. Growth factors for microorganisms. Biochem. Rev., 15: 375.

Steinberg, R.A. 1936. Some effects on the heavy metals essential for the nutrition of Aspergillus niger upon its growth. Am. J. Botany, 23: 227.

Steinberg, R.A. 1939. Growth of fungi in synthetic nutrient solutions. Botanical Rev., 5: 327.

Stewart, R.A., and Meyer, K.F. 1938. Studies in the metabolism of Coccidioides immitis. J. Infect. Dis., 63: 196.

Tatum, E.L. 1944. Biochemistry of fungi. Biochem. Rev., 13: 667.

Van Lanen, J.M. 1947. The absorption of niacin by yeast. Arch. Biochem., 12: 101.

Wickerham, L.J. 1946. A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeast. J. Bact., 52: 293.

Wickerham, L.J. and Burton, K.A. 1948. Carbon assimilation test for the classification of yeast. J. Bact., 56: 363.

Wilders, E. 1901. Nouvelle substance indespensable au developpement de la leure. LaCellule, 18: 313. Cited from Wolf and Wolf "The Fungi." Vol. 2: 24; 1947.

Williams, R.J. and Rohrman, E. 1936. B-alanine and bios. J. Am. Chem. Soc., 58: 695.

Williams, R.J. 1941. Growth-promoting nutrilites for yeast. Biol. Rev., 16: 49.